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A review of conventional and emerging technologies for hydrogels sterilization

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ABSTRACT

Hydrogels are extensively used in the biomedical field, as drug delivery systems, wound dressings, contact lenses or as scaffolds for tissue engineering. Due to their polymeric nature and the presence of high amounts of water in their structure, hydrogels generally present high sensitivity to terminal sterilization. The establishment of an efficient sterilization protocol that does not compromise the functional properties of the hydrogels is one of the challenges faced by researchers when developing a hydrogel for a specific application. Yet, until very recently this aspect was largely ignored in the literature. The present paper reviews the state of literature concerning hydrogels sterilization, compiling the main findings. Conventional terminal sterilization methods (heat sterilization, radiation sterilization, and gas sterilization) as well as emerging sterilization techniques (ozone, supercritical carbon dioxide) are covered. Considerations about aseptic processing are also included. Additionally, and as a framework, hydrogels' polymeric materials, types of networks, and main biomedical applications are summarily described.

1. Introduction

Hydrogels are three-dimensional polymeric networks with the ability to absorb large amounts of water and other biological fluids without dissolving (Mahinroosta et al., 2018; Singhal et al., 2016; Sharma and Tiwari, 2020). Their high-water content, flexibility, and softness make them structurally similar to living tissue. This resemblance, together with general good biocompatibility, makes hydrogels ideal materials for several biomedical and health-related applications such as tissue regeneration, drug delivery, wound dressings, and contact lenses (Caló and Khutoryanskiy, 2015; Hu et al., 2019; Aswathy et al., 2020; Sun et al., 2020).

Sterility is an essential requisite for any biomaterial intended to be implanted or to be in close contact with living tissues (e.g. organs, or to replace part of it such as in bone defects and wounds). Based on the potential applications of hydrogels in the medical and pharmaceutical areas, the requirements from the European Medicines Agency (EMA) for the production of sterile medicinal products and sterile components should be considered (European Medicines Agency (EMA), 2019), as well as the instructions of the European Pharmacopoeia (Ph. Eur.), and/or the Food and Drug Administration (FDA) recommendations. This will depend on the country where the applicant wants to obtain the marketing authorization. The focus of this review is not to address the regulatory procedures related to the sterilization processes, but some considerations will be stated.

Two main approaches can be followed to obtain a sterile product: aseptic processing or terminal sterilization (Galante et al., 2018b). Aseptic processing requires the sterilization of all raw materials and equipment involved in the production, as well as the assurance of operational conditions capable to maintain sterility. This is a costly procedure that requires the maintenance of an extremely controlled production environment and that does not offer the level of security of terminal methods. Thus, aseptic processing is only adopted when the terminal sterilization of the final product is not possible. In terminal sterilization, the finished product is sterilized in its final package. Common (final) sterilization methods include steam and dry heat, ionizing radiation, gas sterilization and sterilizing filtration, which are described in the Ph. Eur (European Pharmacopoeia, 2017). The adoption of this approach reduces the costs of production and enables the achievement of a higher sterility assurance level (SAL), a probabilistic

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parameter used to assess the effectiveness of a sterilization process. A value of SAL $\leq 10^{-6}$, i.e the probability of having not more than one viable microorganism in 10^6 sterilized units of the final product, is usually a critical requirement for health care products and medicines (European Pharmacopoeia, 2017; European Medicines Agency (EMA), 2019). Similar regulation is expected to be applied to hydrogels-based products, intended for medical/pharmaceutical applications (e.g. drug delivery and tissue engineering).

However, exposition to the harsh physical or chemical conditions of the sterilization process can change the chemical, physical, and mechanical properties of the biomaterials or even lead to the formation of toxic residues. Depending on the nature (e.g. aqueous, non-aqueous, semi-solid, etc.) of the products to be sterilized, a different decision tree is available to follow/select the most adequate sterilization procedure (European Medicines Agency (EMA), 2019). The risk of degradation and impurities emergence during sterilization should be assessed.

Sterilization of hydrogels is particularly challenging since the presence of water can enhance and/or enable a series of chemical modifications in the network structure. The sensitivity of hydrogels to some sterilization processes is well documented in the literature (Al-Sabah et al., 2019; Galante et al., 2018a,b; Kanjickal et al., 2009; Tichý et al., 2016; Yao et al., 2020). Nonetheless, each hydrogel is unique in terms of its chemical composition, structure, and properties, being hydrogels classified based on many characteristics such as source, charge, response to stimuli, etc (Table 1). Numerous works discussing hydrogels nature, network, synthesis and properties are available on literature (Bashir et al., 2020; Mahinroosta et al., 2018; Singhal et al., 2016; Sharma and Tiwari, 2020).

Therefore, there is not a universal sterilization method that can be applied to all hydrogels, and studies must be conducted for each system to select the sterilization process (sterilization method and sterilization conditions) that allows the achievement of the required legal SAL and that simultaneously does not significantly alter the main properties of the hydrogel, particularly the ones most relevant for its intended application. Despite the continuous advances made in the development of hydrogels for biomedical applications, namely in terms of materials, fabrication methods, and hydrogels' properties, the sterilization aspect has been seldom addressed (Galante et al., 2018b). However, in the last ~ 20 years, the number of publications regarding this topic has grown and caught the attention of many researchers, especially in the last decade (Fig. 1). Almost 80 % of all papers dealing with "hydrogel" and "sterilization" were published in the last 10 years.

The number of patents regarding the sterilization of hydrogels is also high, more than one hundred thousand results show up when searching for "hydrogel sterilization". Examples such as US9278139B2, US7968050B2, US20160101200A1, US8721963B2, WO198800 3414A1, EP1785153A3, refer the use of radiation in most cases, but also the use of hydrogen peroxide, and supercritical CO_2, for specific hydrogels. Since those patents claim the efficiency of method, they do not describe the effects of sterilization on selected polymers.

In this paper, we present a comprehensive review of the literature concerning hydrogel sterilization studies published in recent years, organized according to the sterilization method, with special attention given to new emerging sterilization methods. These main issues will be preceded by a brief introduction to hydrogels' main biomedical applications.

2. Biomedical applications

Hydrogels have been extensively used in several fields. This brief review will focus on the main biomedical/health applications where their sterilization is crucial (Fig. 2).

Several hydrogels have been proposed as drug delivery systems. Sustained drug release can be achieved by diffusion, degradation of the matrix or external/endogenous triggers, such as changes in temperature, pH, electric and magnetic fields, solvent compositions, light, etc

Table 1
Hydrogel classifications according to some of their features and final/functional properties (Mahinroosta et al., 2018; Singhal et al., 2016; Sharma and Tiwari, 2020) with the nomenclature actualized according to IUPAC (Jones et al., 2008).

Source	**Natural-origin**: comprises biomacromolecules, i.e., macromolecules formed by living organisms (e.g., proteins, nucleic acids, and polysaccharides); **Man-made:** comprises macromolecules that are man-made; **Man-modified**: comprises chemically modified biomacromolecules.
Charge	**Non-charged**: comprised macromolecules having no anionic/cationic charges, or of the zwitterionic type (net charge is zero); **Charged**: comprises macromolecules having anionic or cationic charges (net charge is not zero); **Zwitterionic**: contains both anionic and cationic groups.
Intramolecular structure	**Homopolymers**: derived from one species of monomer; **Copolymers**: derived from more than one species of monomer; **Interpenetrating networks**: comprises two or more networks that are, at least, partially interlaced on a molecular scale but not covalently bonded to each other. These networks cannot be separated unless chemical bonds are broken; **Semi-interpenetrating networks**: comprises one (or more) networks and one (or more) linear or branched polymer(s), and where the latter can penetrate on a molecular scale of, at least, one of the former networks.
Structure	**Amorphous:** comprises polymers that are in the amorphous state, i.e., in a state of matter that is characterized by the absence of long-range molecular order; **Crystalline/semicrystalline:** comprises polymers having a significant fraction of material in the crystalline state, i.e., a state of matter that is ideally characterized by a three-dimensional, long-range order on an atomic scale. It should be noted that polymers rarely crystallize completely and, therefore there is always some amorphous material coexisting with the crystalline phases (thus presenting a specific degree of crystallinity).
Degradability	**Biodegradable:** comprises polymers susceptible to degradation by biological activity (in specific biological environments) by lowering the molar masses of macromolecules that form the substances; **Non-biodegradable:** comprises polymers not susceptible to degradation by biological activity (in specific biological environments) by lowering the molar masses of macromolecules that form the substances.
Crosslinking	**Physically crosslinked**: comprises macromolecules having regions from which, at least, four chains emanate, and which are formed by the intermolecular or intramolecular interactions between existing macromolecules and other molecules that are present (e.g., ionic and electrostatic interactions); **Chemically crosslinked**: comprises macromolecules having regions from which at least four chains emanate, and which are formed by reactions involving sites or groups on existing macromolecules, and other molecules that are present (e.g., covalent bonds); **Permanently crosslinked:** crosslinked polymers (formed by covalent bonds, intermolecular or intramolecular interactions) that are stable under the conditions of use of the material formed; **Transiently crosslinked**: crosslinked polymers (formed by covalent bonds, intermolecular or intramolecular interactions) that are unstable under the conditions of use of the material formed.
Response	**Responsive:** comprised by macromolecules that respond to external electrical, mechanical, thermal, light-induced or chemical stimulation (e.g., pH, ionic strength, electromagnetic field, electrical current, light/radiation, temperature, pressure, stress/shear, enzymes, oxidants/reducers, etc.); **Non-Responsive:** comprised by macromolecules that do not respond to external electrical, mechanical, thermal, light-induced or chemical stimulation (e.g., pH, ionic strength, electromagnetic field, electrical current, light/

(*continued on next page*)

Table 1 (*continued*)

	radiation, temperature, pressure, stress/shear, enzymes, oxidants/reducers, etc.).

(Aswathy et al., 2020; Dreiss, 2020).

Among all polymers and materials used for hydrogel preparation in the biomedical/health area, there is a great interest in chitosan-based hydrogels for sustained drug delivery (Peers et al., 2020). Chitosan-based hydrogels may hinder the diffusion of drugs and consequently their release (e.g. the anti-inflammatory ibuprofen loaded in carboxymethyl-hexanoyl-chitosan structure (Liu and Lin, 2010). Others allow the controlled release of anticancer drugs. Some examples include: doxazocin loaded in chitosan-PVA crosslinked hydrogels (Jamal et al., 2018), and 5-fluorouracil impregnated in chitosan *in situ* gelling hydrogel for injection (Chang et al., 2015).

Injectable hydrogels have attracted more attention in recent years (Sun et al., 2020). In particular, those formed *in situ*, triggered by physiological temperature or by an external stimulus, are an alternative to invasive procedures for implantation (Dreiss, 2020). Other innovative systems for drug delivery include 3D printing of hydrogels, and hydrogel-based microneedles, which have been reviewed elsewhere (Dreiss, 2020).

Another application of hydrogels is their use as contact lenses. Hydrogel contact lenses have a wide range of characteristics, and silicone hydrogel lenses (containing siloxy groups) have a dominant position in the market due to their higher oxygen permeability and comfortable fit (Aswathy et al., 2020; Fan et al., 2020). For contact lenses, to achieve extended drug delivery and higher drug loadings, the addition of Vitamin E as a diffusion barrier, the chemical modification of lenses' surfaces, the incorporation of drug-loaded nanoparticles in the hydrogel structure, or the use of molecular imprinting hydrogels have been proposed (Fan et al., 2020; Pimenta et al., 2017; Yañez et al., 2011; Zhang et al., 2020). Nevertheless, issues like critical lens properties, initial burst release, shelf-life stability, and drug degradation during sterilization and storage are still a challenge and need to be further addressed before commercialization (Fan et al., 2020; Zhang et al., 2020).

Plenty of materials have been produced and are available on the

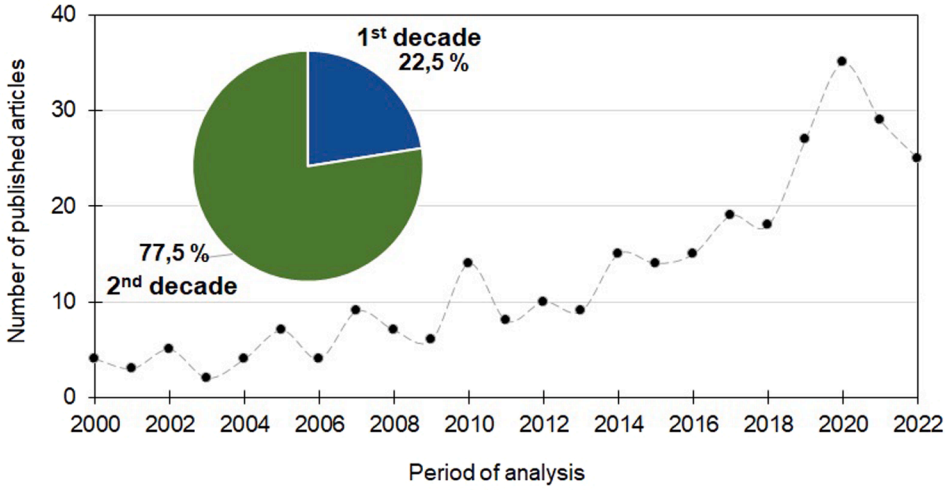

Fig. 1. The number of publications (review and research articles) dealing with hydrogels and sterilization. The literature search was performed in Scopus, for the period of publishing data available (from 2000 up to 2022) using as descriptors "hydrogel" and "sterilization".

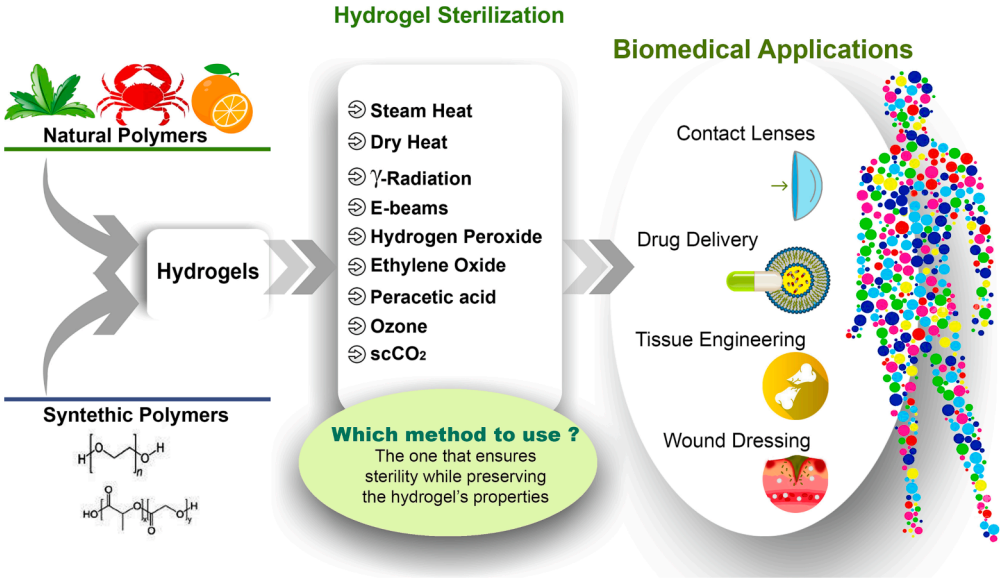

Fig. 2. Main hydrogel sterilization methods and biomedical applications.

market for wound dressing applications. However, not all fulfil the specific requirements of a perfect wound dressing system (Boateng and Catanzano, 2015). One of the advantages of hydrogel-based wound dressings is that it can decrease pain due to a cooling effect and low adherence to the tissue/wound. Hydrogel-based dressings are, in fact, promising systems since they can keep the wound moist and absorb the exudate; avoid adhesion to sensitive underlying tissue, and reduce pain (Koehler et al., 2018). Furthermore, hydrogel dressings may be impregnated with antimicrobial agents and may be used as drug-controlled release systems.

Due to its biocompatibility, low toxicity, and antimicrobial and haemostatic activity, among many others, chitosan has been a material of choice for the development of hydrogels for wound dressing (Koehler et al., 2018; Hamedi et al., 2018). There are already some chitosan-based dressings commercially available but correspond to a low number of systems when compared to the high interest in chitosan for this type of application. Despite that, the presence of chitosan on a wound surface has promoted cell proliferation, and collagen and hyaluronic acid formation (Koehler et al., 2018). Hydrogels containing chitosan and bioactive ingredients have been explored, such as those with antibiotics (e.g. gentamicin, amoxicillin), anti-inflammatory drugs (e.g. ibuprofen) or essential oils (e.g. thyme oil), as recently reviewed (Hamedi et al., 2018).

Alginate is also one of the most frequently used polymers (Koehler et al., 2018). Being hydrophilic, it can easily absorb high volumes of wound exudate, as intended. Moreover, it has a haemostatic effect and increases cell migration.

Hydrogels present ideal characteristics for tissue engineering/regenerative medicine, such as biocompatibility, biodegradability, highly porous structure, high water content, controllable physical properties and flexibility in fabrication (Tran et al., 2020). In addition, hydrogels present structures similar to the extracellular matrix of many tissues and can be delivered in a minimum invasive manner (Morteza Bahram and Moghtader, 2016).

In tissue engineering, hydrogels can be applied as space-filling agents, delivery vehicles for bioactive substances able to influence cellular behaviour or three-dimensional structures that allow cell organization and present stimuli for tissue development (Caló and Khutoryanskiy, 2015; Morteza Bahram and Moghtader, 2016). Space-filling agents are the most common application, where the scaffolds are employed for bulking, to prevent adhesion or act as bioadhesives. Hydrogels for this application must be able to preserve the desired volume and maintain their integrity during the required time (Caló and Khutoryanskiy, 2015; Morteza Bahram and Moghtader, 2016). Hydrogels can also be used to deliver bioactive substances to a target tissue, to promote angiogenesis and encapsulation of secretory cells. A scaffold used as a delivery vehicle allows local and specific delivery to the desired tissue, avoiding the drug degradation or its uptake by other tissues, to which it may be toxic (Caló and Khutoryanskiy, 2015; Morteza Bahram and Moghtader, 2016). In addition, hydrogel scaffolds can be applied for cell transplant and to engineer many tissues, such as cartilage, bone, muscle, fat, liver and neurons. Their highly hydrated three-dimensional network provides an ideal chemical and mechanical environment for cell adhesion, proliferation and differentiation, making them suitable for tissue development (Caló and Khutoryanskiy, 2015; Morteza Bahram and Moghtader, 2016).

3. Hydrogel sterilization

In most biomedical applications, the sterility of the hydrogels is an essential requisite to minimize the risk of infections, one of the biggest health care problems. However, the sterilization of sensitive materials such as hydrogels is one of the most difficult tasks to complete, due to their sensitivity to temperature and radiation and the presence of water in their structure. The high-water content composition is a challenge for sterilization methods since the hydrolysis of the biopolymers is one of the mechanisms of degradation during the processing (Pohan et al., 2020; Bernhardt et al., 2015). Due to this, and whenever the final application permits it, hydrogels are dried before sterilization and after that, they could be re-hydrated to be applied (Kanjickal et al., 2009; Eljarrat-Binstock et al., 2007). However, for some hydrogels formulations/applications, like soft contact lenses or bio-inks, drying is not an option. Therefore, conventional methods, such as steam sterilization, gamma and e-beam irradiation, ethylene oxide (EO), hydrogen peroxide, must be considered and carefully evaluated, or newly available sterilization techniques can be explored. The following methods can be used for sterilization purposes, but before choosing one, it is essential to study how it affects the product to be sterilized and evaluate its efficiency and impact on the product properties, to validate its use (European Pharmacopoeia, 2017; European Medicines Agency (EMA), 2019). It is also necessary to consider the final application of the hydrogel, as not only the material integrity is important, but also, in some cases, such as drug delivery for instance, the drug degradation, stability and release should also be considered.

3.1. Heat sterilization (steam and dry heat)

Steam heat is the most used method of sterilization and the recommended one, if applicable (European Pharmacopoeia, 2017; European Medicines Agency (EMA), 2019). It takes place in an autoclave, combining high temperatures and high humidity that destroy essential cells' metabolic and structural components, killing the microorganisms (European Pharmacopoeia, 2017; Galante et al., 2018b; Russel et al., 1999). This process generally occurs at temperatures between 121 °C and 130 °C for short periods, 15–20 min. This process is usually validated using *Bacillus stearothermophilus* spores as a biologic indicator (European Pharmacopoeia, 2017; European Medicines Agency (EMA), 2019).

Sterilization with dry heat, despite being less efficient, overcomes some of the limitations of steam sterilization related to the presence of water as it uses no water vapour at the expense of higher temperatures (Galante et al., 2018b). Dry heat sterilization occurs at 160 °C for a longer period, about 2 h, yet higher temperatures can be applied (European Pharmacopoeia, 2017; Galante et al., 2018b). In this case, microorganisms are only eliminated due to the heat and chemical oxidation of their constituents, leading to the denaturation of proteins and essential enzymes (Fig. 3). The recommended biological indicator to validate this process are *Bacillus subtilis* spores (European Pharmacopoeia, 2017; Galante et al., 2018b).

The advantages of using heat as a sterilization method lie in efficiency, speed, simplicity, low cost and the non-generation of toxic waste (Fig. 4) (Dai et al., 2016; Galante et al., 2018b). However, the high temperatures used limit the applicability of this method since they affect the structural properties of several biodegradable polymers and in the case of hydrogels can lead to the reduction of water content due to evaporation (Beard et al., 2021) (Table 2). Additionally, the presence of water vapor can cause the degradation and hydrolysis of some polymers, conditioning their structure and surface, and decreasing the biocompatibility of the materials (Dai et al., 2016; Ahmed et al., 2013; Tessarolo, 2008).

Tichý et al. (Tichý et al., 2016) evaluated the use of steam heat as a sterilization method for several polymeric hydrogels, of natural and synthetic origin, in the presence and absence of electrolytes. For synthetic hydrogels, the authors identified that, in the presence of ions, sterilization did not significantly affect hydrogel properties. In the case of natural-based hydrogels, steam sterilization had a significant impact on hydroxypropyl methylcellulose hydrogels, causing a decrease in viscosity, hardness and adhesiveness, even after storage. For xanthan gum hydrogels and tragacanth gum hydrogels, a decrease in most of the evaluated parameters was observed, and for agar hydrogels, it was observed an increase in viscosity, compressibility and hardness after sterilization.

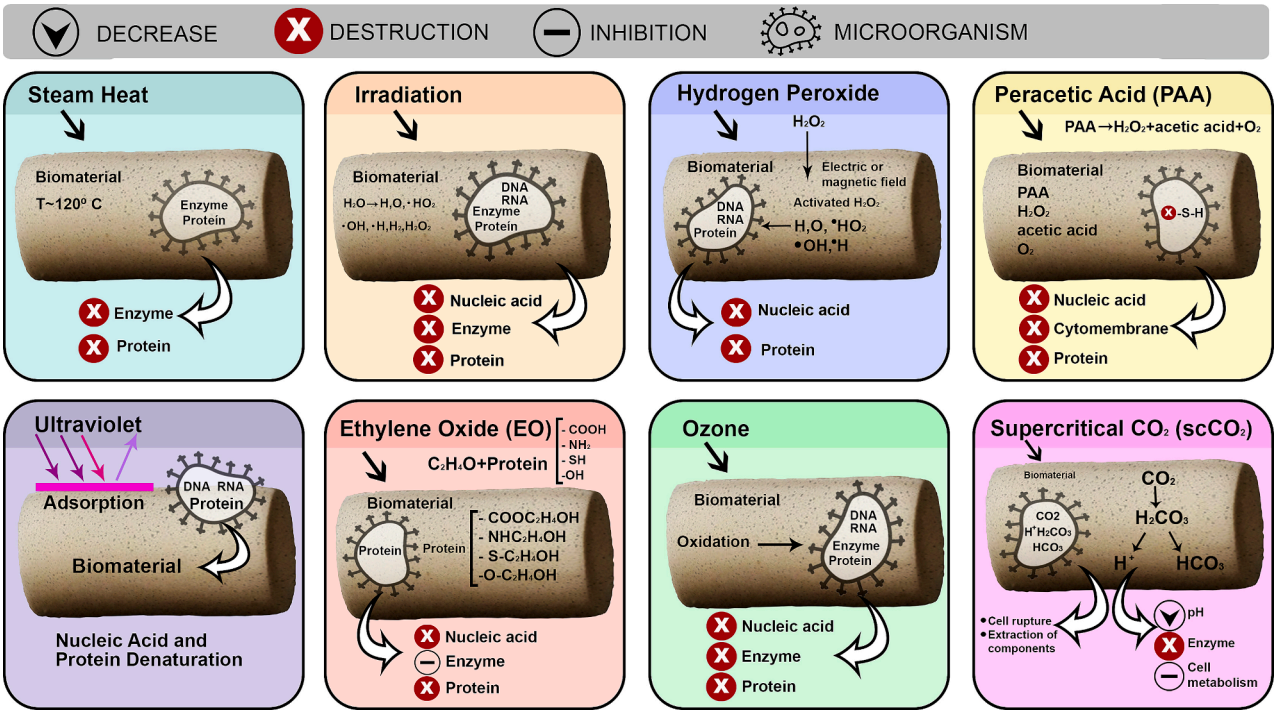

Fig. 3. Main sterilization mechanisms of selected methods used on hydrogels. Figure based on Tao et al., (2021) (Geiger et al., 2003).

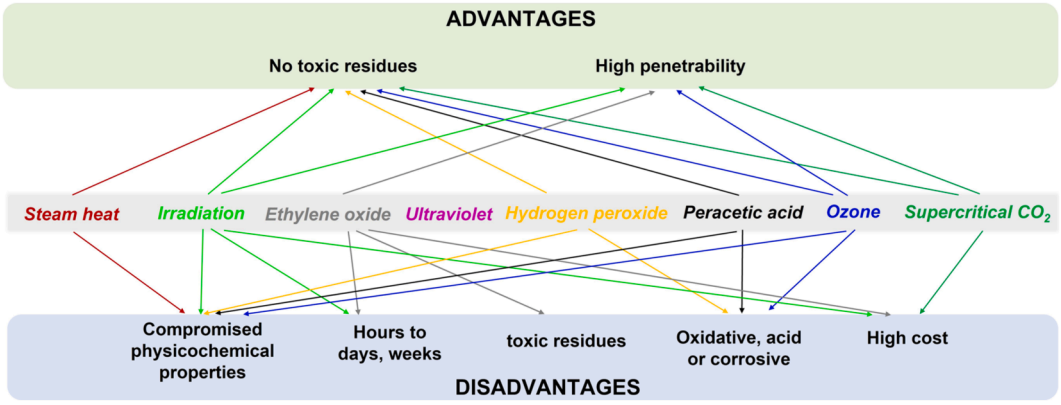

Fig. 4. Advantages and disadvantages of sterilization methods.

In their work, Stoppel et al. (Stoppel et al., 2014) showed that steam sterilization causes the loss of mechanical properties and a decrease in the swelling rate of alginate hydrogels, indicating possible changes in the network due to the sterilization treatment .

Haridas et al. (Haridas and Rosemary, 2019) produced hyaluronic acid (HA) hydrogels and tested steam heat as a sterilization method for these materials, varying sterilization temperature and time. The hydrogels showed large variations in viscoelasticity and lost transparency when autoclaved for 15 min at 121 °C. Yet, when slightly lower temperatures were evaluated, such as 120 °C to 118 °C, and the samples were sterilized for 30 min, degradation occurred. Porosity also changed after sterilization and an increase in pore size was observed, despite the swelling capacity remaining the same. Szabó et al. (Szabó et al., 2013) also tested the effect of steam sterilization on HA hydrogels and verified structure degradation and changes in viscoelasticity. However, they concluded that by controlling the HA concentration the impact of steam sterilization may be minimized.

Al-Sabah et al. (Al-Sabah et al., 2019) showed in their studies that steam sterilization produced changes in the surface morphology, pore size and mechanical properties of sodium alginate and nanocellulose-based hydrogels.

Rizwan et al. (Rizwan et al., 2020)sterilized gelatin methacryloyl hydrogels using different methodologies, among them steam sterilization. Results indicated that the mechanical properties were compromised by the sterilization, as a decrease of the compressive modulus occurred. Furthermore, the swelling capacity suffered an increase.

Henise et al. (Henise et al., 2020)were able to successfully sterilize by autoclaving a tetra-polyethylene glycol hydrogel with crosslinks containing a carbamate group susceptible to suffer cleavage by a β-elimination reaction, controlled by pH. The authors concluded that by lowering the pH the hydrogel structure remained unaffected, occurring with sterilization no significant cleavage of the crosslinkers. In their study, Karajanagi et al. (Karajanagi et al., 2011)studied the effect of steam sterilization on PEG hydrogels and identified changes in the hydrogel's morphology.

Galante et al. (2018) (Galante et al., 2018a) studied the influence of steam sterilization on drug-eluting silicone hydrogels and concluded that, despite causing changes in some mechanical properties and causing

Table 2
Sterilization methods and their impact on hydrogel properties.

Sterilization Method	Hydrogel Composition	Sterilization Impact	Reference
Steam heat	Carrageenan, xanthan gum, tragacanth gum; agar; methylcellulose, hydroxypropyl methylcellulose and sodium carboxymethylcellulose; Poly(acrylic acids) (linear or crosslinked); poly(vinylmethylether) (PVM)/Maleic acid (MA) decadiene.	Changes in viscosity, hardness, compressibility adhesiveness.	(Tichý et al., 2016)
	Alginate	Loss of mechanical properties and decrease of the swelling rate.	(Stoppel et al., 2014)
	Hyaluronic acid/sodium hyaluronate	Enzymatic and structural degradation and changes in viscoelasticity and swelling properties.	(Haridas and Rosemary, 2019; Szabó et al., 2013)
	Sodium alginate and nanocellulose	Changes in surface morphology, pore size and mechanical properties.	(Al-Sabah et al., 2019)
	Chitosan	Chemical degradation	(Galante et al., 2016)
	Gelan gum	No significant changes in weight or rheological properties.	(Leone et al., 2020)
	Gelatin methacryloyl	Decrease in mechanical properties; swelling capacity increase.	(Rizwan et al., 2020)
	Lignocellulosic components from Sisal fibers	No changes in physico-mechanical properties.	(Queiroz et al., 2021)
	Poly(ethylene)-glycol (PEG)	Morphological changes or no changes in size/ appearance.	(Henise et al., 2020; Karajanagi et al., 2011)
	Silicone (TRIS)	Changes in mechanical properties and decrease in drug release; increase in the swelling capacity.	(Galante et al., 2017, 2018a)
	Pluronic® F127	Loss of gelation properties; increase in viscosity. Increased polymer weight fraction; decrease in gelation temperature	(Beard et al., 2021; Rafael et al., 2019)
Irradiation	Agarose	Decrease in viscoelastic properties ; radiolytic degradation; chemical changes; network damage and mechanical instability.	(Krömmelbein et al., 2021)
	Gelatin and collagen	Chemical degradation and crosslinking.	(Hara et al., 2010)
	Chitosan	Possible chemical degradation; increase in particle size and polydispersity; chemical changes.	(Galante et al., 2016)
	Alginate and gelatin	Decrease in swelling properties; chemical degradation.	(Fu et al., 2021)
	Gelatin methacryloyl	Increase in mechanical properties; swelling capacity decrease; decrease in pore size and biodegradation.	(Rizwan et al., 2020)
	Poly(ethylene)-glycol (PEG)	Compromised structure; formation of free radicals.	(Kanjickal et al., 2009; Karajanagi et al., 2011)
	CyborGel™ (proprietary hydrogel)	Chemical changes.	(Tohfafarosh et al., 2016)
	Poly(vinyl alcohol)/poly (vinyl pyrrolidone) (PVA/PVP)	Changes in mechanical properties; decrease in surface hydrophilicity and increase in cell adhesion and proliferation.	(Yao et al., 2020; Shi et al., 2014)
	Hydroxyethyl methacrylate (HEMA) crosslinked with ethylene glycol dimethacrylate (EGDMA)	Decrease in water adsorption capacity; no changes in pore size and shape; increase in elastic modulus.	(Eljarrat-Binstock et al., 2007)
Ethylene oxide	Gelatin methacryloyl	Decrease in mechanical properties; swelling capacity increase.	(Rizwan et al., 2020)
	Poly(ethylene)-glycol (PEG)	Swelling capacity decreases.	(Kanjickal et al., 2009; Kanjickal et al., 2008)
	Hydroxyethyl methacrylate (HEMA) crosslinked with ethylene glycol dimethacrylate (EGDMA)	Density increases; drug loading decreases and changes to the mechanical properties.	(Eljarrat-Binstock et al., 2007)
	Poly(lactic-*co*-glycolic acid) (PLGA) and collagen	Chemical changes and mechanical properties are compromised.	(Geiger et al., 2003)
Hydrogen peroxide	Poly(ethylene)-glycol (PEG)	Swelling capacity increases, changes in the surface structure and free radical concentration increase	(Kanjickal et al., 2009; Kanjickal et al., 2008)
Ozone	Silicone (TRIS)	Ionic permeability increase; changes in the mechanical properties, surface morphology and topography; possible chemical degradation; Changes in swelling capacity and thermomechanical properties.	(Galante et al., 2017, 2018a)
	Chitosan	No changes in morphology or conductivity, size	(Galante et al., 2016)

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Table 2 (*continued*)

Sterilization Method	Hydrogel Composition	Sterilization Impact	Reference
		decrease and some toxicity.	
Supercritical CO_2	Alginate and collagen	Changes in mechanical and rheological properties.	(Bernhardt et al., 2015)
	Alginate/gelatine	No significant changes in chemical and calorimetric properties, density or appearance. For some sterilization conditions, the mechanical properties, pore volume and surface area were affected.	(Bento et al., 2022)
	Poly(acrylic acid-*co*-acrylamide	No significant changes in sweeling properties or structure.	(Jiménez et al., 2008)
	Poly(ethylene)-glycol (PEG)	No changes in appearance, viscoelastic properties or water-binding capacity.	(Karajanagi et al., 2011)
	Polyurethane	No changes in physico-chemical properties and water content.	(Garle et al., 2020)

The literature search was performed in Scopus, for the period of publishing data available (from 2000 up to 2021) using as descriptors "hydrogel" and "sterilization".

a decrease in drug release, it was the least aggressive sterilization method among the tested ones. In other work, they observed steam sterilization caused an increase in the swelling capacity of silicone hydrogels and, for chitosan hydrogel nanoparticles, the steam sterilization method promoted the degradation of the samples. (Galante et al., 2016).

Rafael et al. (Rafael et al., 2019) produced thermo-sensitive hydrogels based on Pluronic® F127 loaded with silver nanoparticles (AgNPs) and tested autoclaving and dry heat as sterilization methods. The hydrogels showed more sensitivity to the sterilization with dry heat, which triggered the loss of gelation properties, while autoclaving had no significant impact on those properties, but caused an increase in the hydrogel's viscosity. None of the sterilization processes affected the antimicrobial properties of AgNPs. The authors concluded that the more suitable method to sterilize the produced hydrogels was autoclaving. Beard et al. (Beard et al., 2021) also sterilized hydrogels with the same composition and identified the reduction of water content of the hydrogel due to evaporation during autoclaving. The gelation temperature also reduced, while other rheological properties and the capacity to work as a delivery vehicle were preserved.

3.2. Radiation sterilization (gamma irradiation, e-beams, ultraviolet)

Another method, widely used as an alternative method of sterilization for heat-sensitive materials in the medical and pharmaceutical field is ionizing radiation, in the form of gamma rays (γ) or electron beams (e-beams) (European Pharmacopoeia, 2017; European Medicines Agency (EMA), 2019). Gamma irradiation comes from radioactive isotopes, such as Cobalt 60, while electron beams are produced by particle accelerators. The standard radiation dose is 25 kGy and the sterilization occurs either by direct ionization of vital cell molecules, such as DNA, or indirectly by the reaction of free radicals produced in the cell fluid, with additional damage to cell membranes and enzymes involved in nucleic acid repair (Fig. 3) (Russel et al., 1999; Pelczar et al., 1986). *Bacillus pumilus* spores are the recommended biological indicator for this method (European Pharmacopoeia, 2017; Galante et al., 2018b).

Despite its advantages, such as high penetrability and the fact that leaves no toxic residues (Fig. 4), this method presents some drawbacks, as some materials can deteriorate when irradiated, especially polymers, in which ionizing radiation can cause breakage of bonds, crosslinking or photooxidation reactions (Galante et al., 2018b). This method is associated with chemical changes in sterilized materials, a decrease in polymer molecular weight, loss of mechanical properties (e.g. tensile strength and modulus of elasticity), transparency, and degradation (Dai et al., 2016; Tessarolo, 2008; Noah et al., 2002). In the case of hydrogels, the presence of water may also have a role, as free OH• and H• radicals may be form due to water radiolysis, which can cause chemical changes in the polymers (Krömmelbein et al., 2021). Also, this method requires several hours to be effective and has high costs associated (Tao et al., 2021).

In their work, Krömmelbein et al. (Krömmelbein et al., 2021) sterilized agarose hydrogels using e-beam radiation. They verified that a decrease in viscoelastic properties occurs and that this decrease was more evident with increasing electron dose. The decrease in this parameter indicated radiolitic degradation. The swelling ratio also increased with increasing electron dose but remained constant after 10 kGy. Chemical changes were also observed due to this sterilization treatment, which lead to a damaged network and mechanical instability.

Rizwan et al. (Rizwan et al., 2020) characterized the effects of gamma irradiation sterilization on gelatin methacryloyl hydrogels. The mechanical properties were changed due to sterilization, namely, an increase in the compressive modulus was observed. Contrarily, the swelling capacity decreased, as well as the pore size. Gamma irradiation led to an increase in crosslinking that resulted in smaller pores and slower biodegradation.

Fu et al. (Fu et al., 2021) tested gamma irradiation for sterilization of alginate-gelatin hydrogel spheres. Results indicated that the physico-chemical properties of the spheres remained intact, however, hydrogels' network debonding and recrosslinking occurred, causing a decrease in water absorption and a higher initial degradation rate.

Karajanagi et al. (Karajanagi et al., 2011) sterilized PEG hydrogels with gamma irradiation, which compromised the hydrogels' structure. Kanjickal et al. (Kanjickal et al., 2009) also studied PEG hydrogels and evaluated the formation of free radicals after gamma irradiation exposure in comparison to other radiation methods, revealing that gamma irradiation contributed to a higher concentration of free radicals.

In their studies, Tohfafarosh et al. (Tohfafarosh et al., 2016) tested the use of gamma and e-beam radiation to sterilize a proprietary hydrogel (CyborGel™). Their results showed that the swelling ratio, chemical, mechanical and tribological properties were not compromised, neither by gamma irradiation nor by e-beam radiation. However, e-beam radiation caused a slight change in the FTIR spectrum of the sample.

Shi et al. (Shi et al., 2014) used gamma radiation to sterilise poly (vinyl alcohol)/poly(vinyl pyrrolidone) (PVA/PVP) hydrogels Their results indicated that both mechanical and tribological properties are radiation dose-dependent, The compressive strength and the compressive modulus increased with the radiation dose, but for doses higher than 100 kGy, a decrease was observed.

Yao et al. (Yao et al., 2020) sterilized PVA hydrogels using gamma irradiation. The results indicated that the sterilization did not affect mechanical properties, however, it caused a decrease in surface hydrophilicity and an increase in cell adhesion and proliferation. The hemocompatibility of the hydrogels was preserved.

Eljarrat-Binstock et al. (Eljarrat-Binstock et al., 2007) produced hydrogel sponges (70 % of water) of hydroxyethyl methacrylate (HEMA) crosslinked with ethylene glycol dimethacrylate (EGDMA) and found gamma irradiation to be suitable for the sterilization of the dried

hydrogels. Irradiation only led to a small decrease in water uptake capacity and a small increase in the elastic modulus, resulting in stiffer hydrogels.

Gamma irradiation of fish gelatine, porcine gelatine, and porcine collagen hydrogels was studied by Hara et al. (Hara et al., 2010) . At low doses of radiation, degradation and crosslinking were verified for all formulations. However, for higher concentrations of the polymer, the effect of the radiation decreases.

Galante et al. (Galante et al., 2016) sterilized chitosan hydrogel nanoparticles using gamma irradiation. The results indicated a possible degradation of the samples, an increase in average particle size and polydispersity and a decrease in zeta potential. Chemical changes were also observed. Conductivity and pH were not affected. The presence of protective sugars (glucose and mannitol 5 %) increased the nanoparticle's resistance to radiation.

Another type of radiation, which can be used in sterilization, is ultraviolet (UV). The main disadvantage of this type of radiation is the weak ability to penetrate matter, eliminating only microorganisms on the surface of the materials. In addition, long exposure times can result in the loss of structural and chemical properties, such as reduced molecular weight and breakdown stress, due to crosslinking and/or chain splitting. UV radiation eliminates microorganisms by being absorbed by various cellular components, namely nucleic acids, with DNA being the main target (Fig. 3).

Stoppel et al. (Stoppel et al., 2014) evaluated the use of UV radiation as a sterilization method for alginate hydrogels and despite not affecting the mechanical properties of the hydrogels, the sterilization of 1 mm tick hydrogels was not successful. However, Yu et al. (Yu et al., 2017) also evaluated the use of UV radiation as a sterilization treatment and concluded that this can be an effective method to sterilize alginate powder for hydrogel production, without compromising its properties.

3.3. Gas sterilization (ethylene oxide, hydrogen peroxide, peracetic acid)

Gas sterilization is only used when the mentioned above sterilization methods are not applicable (European Pharmacopoeia, 2017; European Medicines Agency (EMA), 2019). For this method, it is necessary to ensure that no gas residues are left behind after sterilization, considering their general toxicity. Due to this, drying of the hydrogel is required, as the toxic residues may be retained in the water (Karajanagi et al., 2011). Typically used gases are ethylene oxide, hydrogen peroxide, and peracetic acid, among others (European Pharmacopoeia, 2017; Galante et al., 2018b). Ethylene oxide (EO) is regularly used, as an alternative to moist heat, in the sterilization of medical devices that cannot withstand high temperatures. The gas causes irreversible alkylation reactions in cell molecules, resulting in changes in cell metabolism, and denaturation of proteins, enzymes and nucleic acids (Fig. 3) (Russel et al., 1999; Pelczar et al., 1986). The efficiency of sterilization depends on several factors, such as exposure time, concentration, temperature and humidity (Russel et al., 1999). In addition, the size of the material to be sterilized, its conditioning and affinity for the gas can influence sterilization. Sterilization takes place in closed stainless steel chambers, for several hours, at temperatures between 40 and 50 °C and relative humidity between 40 and 80 %, with a gas concentration between 400 and 1000 mg/L, in a vacuum or under pressure (Russel et al., 1999; Dai et al., 2016). Despite the advantages it presents, such as efficiency, good penetration ability and compatibility with various materials, this gas also has its disadvantages (Fig. 4). It can affect the properties of some biodegradable biopolymers, as it can cause changes in mechanical and chemical properties, molecular weight and degree of degradation after sterilization (Table 2) (Dai et al., 2016; Tessarolo, 2008). In addition, the gas needs to be completely removed, which requires several days and temperatures of 40 °C, in the case of porous materials. Due to its high reactivity, it can present toxic risks associated with itself and its derivatives, formed during the sterilization process (Zhang et al., 2006). To validate the use of this gas for sterilization purposes it is recommended the use of *Bacillus subtilis* spores (Galante et al., 2018b).

Hydrogen peroxide (H_2O_2) has known microbicidal effects and can be used in gas or plasma form. Its action involves the formation of hydroxyl radicals, which are strong oxidizers, reacting with metal ions present in the cells, the phospholipids of the cellular membrane, DNA, and other essential components that have double bonds (Fig. 3) (Russel et al., 1999). Sterilization with hydrogen peroxide vapor is not frequent, being more common in the food industry or as a surface sterilization technique for medical devices (Russel et al., 1999).

Rizwan et al. (Rizwan et al., 2020) also characterized the effects of EO sterilization on gelatin methacryloyl hydrogels. The results showed a decrease in the compressive modulus and an increase in the swelling capacity due to EO sterilization, similarly to what happened with steam sterilization. Kanjickal et al. (Kanjickal et al., 2009) sterilized PEG hydrogels with EO and H_2O_2 to evaluate how these processes affect the free radical concentration in the samples. The sterilization with EO did not seem to affect the free radical concentration, contrarily to H_2O_2, which increased the concentration of free radicals. This increase may cause a change in the hydrogel's properties over time. In another work, they tested the effect of EO and H_2O_2 on PEG hydrogels for drug delivery (Kanjickal et al., 2008). EO led to a reduction in the swelling capacity, while H_2O_2 led to an increase in this parameter. Also, H_2O_2 produced changes in the surface morphology and caused an increase in the free radical concentrations.

Eljarrat-Binstock et al. (Eljarrat-Binstock et al., 2007) prepared hydrogel sponges of hydroxyethyl methacrylate (HEMA) crosslinked with ethylene glycol dimethacrylate (EGDMA) for drug delivery and tested the use of EO as a sterilization method. EO led to an increase in density, a decrease in the drug loading and changes in the mechanical properties. The authors identified that the shape of the hydrogel is an important parameter in sterilization since cylindrical hydrogels were more sensitive to EO sterilization than rectangular hydrogels.

Peracetic acid (PAA) is used as a sterilization agent thanks to its relatively high penetrating power and inactivation efficiency of various microorganisms (Russel et al., 1999). This compound has been used in the food industry and healthcare, in the sterilization of heat-sensitive medical devices. A major advantage of PAA is its degradation into components that do not have an environmental impact. However, it is a corrosive gas and is therefore not widely used (Fig. 4). Like hydrogen peroxide, peracetic acid forms hydroxyl radicals, which are responsible for inactivating microorganisms by attacking cells' vital components (Fig. 3) (Russel et al., 1999; Dai et al., 2016). Peracetic acid affects the structural properties of biodegradable materials, due to the established oxidative and acidic environment. In addition, it leaves acid residues behind that raise concerns regarding biocompatibility (Dai et al., 2016). No works regarding the sterilization of hydrogels using this gas were found in the literature, yet in some works, its use as a disinfection method is evaluated (Wirtanen et al., 2001; Harkonen et al., 1999).

3.4. New emerging techniques (ozone, supercritical CO_2)

Ozone sterilization is a newly emerging technique with the potential to sterilize medical devices (Galante et al., 2018b). This gas has strong oxidative powers that can inactivate the microorganisms (Fig. 3). The process occurs at low temperatures making it suitable for heat-sensitive materials, and gas concentration, humidity and processing time can be adapted to the type of material to be sterilized. Besides, the gas has a great penetration power when compared to others and has no toxic residues associated (Fig. 4) (Galante et al., 2018b). Yet, in some cases it can lead to degradation due to oxidative reactions (Dai et al., 2016).

Galante et al. (Galante et al., 2017) tested the use of ozone as a sterilization method for silicone-based hydrogels. Ozone was able to effectively sterilize the hydrogels while preserving the properties of interest for the intended application. However, for a higher exposure to ozone there was an increase in the ionic permeability and friction coefficient. Moreover, the mechanical properties, surface morphology and

topography were compromised, and a possible degradation occurred. In another work, they evaluated the impact of this sterilization method on drug-loaded silicone hydrogels (Galante et al., 2018a). The sterilization affected the swelling capacity and the thermomechanical properties and led to drug degradation.

In another work, Galante et al. (Galante et al., 2016) tested the use of ozone as a sterilization method for chitosan hydrogel nanoparticles. The properties of the samples were not affected. However, ozone sterilization was not as effective as gamma irradiation and appeared to originate some toxicity. The addition of protective sugars caused chemical changes after ozonation.

The use of supercritical CO_2 ($scCO_2$) as a sterilization agent has a high potential for processing temperature-sensitive materials, despite not being approved by the European entities as a recommended sterilization method. Carbon dioxide in the supercritical state has a low viscosity and zero surface tension, which gives it a high penetration capacity in complex and porous structures (Fig. 4). This gas leaves no toxic residues behind and the only disadvantage associated with this method is its high cost. Several mechanisms have been proposed to explain the deactivation of microorganisms by $scCO_2$, which can all occur simultaneously: rupture of the cell, denaturation of essential enzymes, acidification of the medium and the extraction of intracellular components by CO_2 (Fig. 3) (Zhang et al., 2006). Different microorganisms can have distinct responses to CO_2 and therefore be inactivated differently. The required deactivation time can vary from minutes to several days, depending on the type of microorganism or the used conditions. Temperature and pressure also have an influence (Zhang et al., 2006; Russell et al., 2015; Garcia-Gonzalez et al., 2007). The use of additives, such as acetic acid, hydrogen peroxide, ethanol or water, in conjunction with carbon dioxide can assist in inactivating microorganisms, allowing lower temperatures and shorter exposure times to be used. Studies reveal that the use of carbon dioxide in the supercritical state as a sterilization method does not significantly affect the mechanical and chemical properties of the materials, maintaining their structure (Table 2). There was also no degradation in the studied biopolymers (Dai et al., 2016; Tessarolo, 2008; Russell et al., 2015). Yet, the use of this method for hydrogels must be done with care, considering that in the presence of water, carbon dioxide may form carbonic acid and reduce the pH of the medium (Raman et al., 2015).

Bernhardt et al. (Bernhardt et al., 2015) tested the use of $scCO_2$, and small concentrations of additives, as a sterilization technique for cylindrical alginate hydrogels and collagen-based biomaterials. They tested the procedure with a wide range of microorganisms and sterilization was successful for most of them. The mechanical and rheological properties were less affected by the $scCO_2$ sterilization compared to other methods like gamma radiation and steam sterilization.

Garle et al. (Garle et al., 2020) were able to successfully sterilize nanoporous polyurethane hydrogel membranes using $scCO_2$ and did not identify any changes caused by the sterilization treatment.

Jiménez et al. (Jiménez et al., 2008) evaluated the sterilization of poly(acrylic acid-*co*-acrylamide) hydrogels with $scCO_2$ alone and with H_2O_2 as an additive. There were no significant changes in the hydrogel properties after treatment and the use of $scCO_2$ with and without additives was proven efficient in the inactivation of microorganisms for the reported experiment conditions, 4 h, 40 °C and 27.6 MPa.

Karajanagi et al. (Karajanagi et al., 2011) showed in their studies that it is possible to sterilize PEG hydrogels with dense CO_2, without compromising their morphology, water content, viscoelastic properties, mechanical properties and biocompatibility, contrarily to steam sterilization and gamma irradiation, which compromised the hydrogels' structure.

In the case that none of the methods mentioned is suitable as a final sterilization method for the product, filtration sterilization of the starting materials and reagents may be performed (European Pharmacopoeia, 2017; European Medicines Agency (EMA), 2019). This type of sterilization requires additional precautions to minimize the possibility of posterior contamination. All the steps after sterilization need to be performed in aseptic conditions.

3.5. Aseptic processing

When a biomedical or pharmaceutical application is intended, the choice of a final sterilization method or the aseptic processing for hydrogel production should be justified. Specific guidelines/instructions from EMA, Ph. Eur. or FDA should be followed to fulfil the requirements for a sterile medicinal product. As previously referred to in this review, aseptic processing is only the choice when the final package/product is not possible to sterilize by final sterilization methods (already discussed). In this case, the ISO 13408–1:2008 standard should be followed (ISO, 2008). This standard "specifies the general requirements for, and offers guidance on, processes, programmes and procedures for development, validation and routine control of the manufacturing process for aseptically-processed health care products".

Aseptic processing is a complex procedure that requires high standards of hygiene and cleanliness as well as specific training of the personnel working in clean areas. In-process controls and validation data for the selected method should be also provided.

Note that the bioburden control criteria must be detailed before all sterilization processes. The bioburden level of the final product (e.g. hydrogel) may be impacted by the microbiological properties of the individual components such as the active substance, excipients, other materials, and/ or containers (European Medicines Agency (EMA), 2019). However, for terminal sterilization, a well-defined process of known lethality delivered to the product and a SAL can be achieved. In the case of aseptic processing, it is not possible and the pre-sterilization of the product, product parts and/or components and all equipment coming into direct contact with the aseptically-processed product is required (ISO, 2008).

One example of aseptic processing application is the development of advanced therapy medicinal products (based on genes, tissues or cells), which usually cannot be terminally sterilized. Therefore, the manufacturing process should be carried out in aseptic conditions (EudraLex, 2017).

Other examples include aseptic handling, transfer and packaging of solid medical devices, and tissues or biological production systems (ISO, 2008). Other products such as wound dressing with hemostatic drugs, transdermal or injectable drug delivery systems, and implants, among others, have also to be aseptically processed (ISO, 2012). A similar procedure may be applied to obtain sterile hydrogels. As an example, Henise et al. (Henise et al., 2020) were able to successfully prepare injectable Tetra-PEG hydrogel microspheres for drug delivery under aseptic conditions. The work included the development of equipment and procedures for aseptic production at large-scale to support clinical development (Henise et al., 2020).

4. Conclusions

Hydrogels present ideal properties for application in the biomedical field. They have been successfully applied in tissue engineering, wound dressing, drug delivery and contact lenses.

Nevertheless, their application in biomedicine requires proper sterilization, which is a challenge for the available technologies. Due to hydrogels' nature, they present a high sensitivity to terminal sterilization. Commonly used sterilization techniques may compromise hydrogels properties such as aspect, colour, chemical structure, swelling behaviour, viscosity and mechanical properties. Novel sterilization techniques seem promising, namely $scCO_2$, that presents a low impact on hydrogels' properties. However, more studies are necessary to validate its use and obtain approval from regulatory authorities. Each method has its drawbacks and the temperature, pressure, dose, time and atmosphere used for sterilization and the possible toxic residues and radicals formed, as well as the nature of the polymers are some of the

factors that need to be considered.

In the case that none of the sterilization methodologies is efficient, aseptic processing should be considered, despite being more complex and requiring a more rigorous control.

More studies on the sterilization impact on the hydrogel's properties are necessary. Not only the impact on properties should be evaluated but also the sterilization efficiency should be tested. The formulations should be tested case-by-case, evaluating sterilization impact and efficiency, to choose the most suitable sterilization method for each, the one that guarantees effective sterilization and does not compromise the intended properties.

Overall, research indicates that all sterilization techniques can cause chemical and physical modifications on the hydrogel's networks (ex: polymer chain breaking or decrosslinking/crosslinking, crosslinks reorganization, side groups modification, etc), impacting properties (such as mechanical and rheological properties, degradation rate, swelling rate, etc) that ultimate can invalidate hydrogels intended applications or even lead to hydrogel's physical disintegration. Notwithstanding, each sterilization technique affects differently each type of hydrogels, depending on the hydrogels' polymer(s) constituents, nature of crosslinks and network architecture, geometry, morphology, etc. In this way, for a particular hydrogel, several methods must be tested to choose the most adequate one and operational conditions should be optimized.

Considering the relatively small number of studies dedicated to hydrogel sterilization currently is still difficult to draw some general trends that could help guide the selection of a specific sterilization method.

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Author contributions

MEMB conceived the idea of a comprehensive review considering hydrogel sterilization, supervised the work, managed the project and MEMB was responsible for funding acquisition. CSAB, MCG, PC and MEMB wrote the manuscript, and H.C.S. reviewed and edited the text with the co-authors. All authors contributed to the revision of the information and have read and approved the final version.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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